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Lutetium Speciation and Toxicity in a Microbial Bioassay: Testing the Free-Ion Model for Lanthanides

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The validity of the free-ion model (FIM) for the element lutetium (Lu), a member of the lanthanides, was assessed in experiments with the bacterium *Vibrio fischeri*. The FIM is mainly based on divalent metals and synthetic ligands and has not yet been validated for the trivalent lanthanides. The bioluminescence response of *V. fischeri* was studied at different Lu concentrations in the presence and absence of natural and synthetic organic ligands [citrate, malate, oxalate, acetate, ethylenediaminetetraacetate (EDTA), and nitrilotriacetate (NTA)]. All ligands were tested separately to ensure that their concentrations would not cause adverse effects themselves. Free Lu^{3+} concentrations were calculated with a speciation program, after extension of its database with the relevant Lu equilibria. The results confirmed the FIM for Lu: that is, in contrast to total dissolved Lu concentrations, free Lu^{3+} concentrations had an apparent relationship with the response of *V. fischeri*. However, a contribution of minor inorganic Lu complexes cannot be ruled out. In the presence of malate and oxalate, the EC_{50} for Lu^{3+} decreased faster in time than for the other ligands, indicating lower elimination rates. With an EC_{50} of $1.57 \mu\text{M}$, Lu^{3+} is more toxic than La^{3+} , Cd^{2+} , or Zn^{2+} and approximately equally as toxic as Cu^{2+} . Although the pH increased slightly during the experiments, it was shown that the influence of pH on Lu speciation was limited.

Introduction

The aqueous speciation of a metal, that is, the different physicochemical forms of a metal that together make up its total concentration in solution (1), has great consequences for its availability and effects on aquatic organisms (2). The ground-breaking work of Sunda and Guillard (3) showed that copper toxicity to phytoplankton was best explained by Cu^{2+}

activities. Such well-defined studies with mainly synthetic ligands [e.g., nitrilotriacetate (NTA) and ethylenediaminetetraacetate (EDTA)] and divalent metals (e.g., Cd, Cu, and Zn) led to formulation of the free-ion model (FIM) or free-ion activity model (FIAM), which states that uptake and toxicity of cationic trace metals are best correlated to free-ion activities (4). According to Campbell (5), the biological response elicited by a dissolved metal is usually a function of the (hydrated) free-metal ion concentration, $\text{M}^{z+}(\text{H}_2\text{O})_n$. Note that the former definition refers to activity and the latter to concentration. Extensions of the FIM include the incorporation of metal speciation dynamics, which is especially relevant in case of labile metal complexes that may supply the free ion (6, 7), and further by considering the binding of metals to biological ligands: the biotic ligand model (BLM) (8). Testing the FIM and possibly extending its application to include trivalent metals, such as the lanthanides, especially in the presence of natural organic ligands, contributes to the understanding and generalization of availability concepts in aquatic toxicology. These types of evaluations are needed to estimate the risks involved with lanthanide emissions to freshwater.

Studying and testing the FIM was greatly enhanced by the development of commercially available speciation modeling software and the collection and evaluation of equilibrium constants for metals with all kinds of (in)organic ligands, notably, the Critically Selected Stability Constants of Metal Complexes Database by Martell and Smith (9, 10).

In general, there are three types of approaches to assess the speciation of a metal: (1) analytical chemical methods, (2) biological methods, and (3) computational methods. Chemical methods often suffer from problems with detection limits, most notably for ambient concentration levels. In addition, the quality control of analytical measurements is often a problem, since chemical equilibria are easily disturbed. Biological methods (such as bioassays) give in fact the most relevant information for environmental research [e.g., Timmermans et al. (11), who used diatoms as iron speciation monitors], but unfortunately these methods do not give a quantitative insight into the concentration nor into the exact identity of the toxic substance. Computation of the speciation may be a suitable approach, provided that relevant equilibria are sufficiently well characterized, including the equilibrium constants (12). For synthetic solutions this is usually the case. Computation is also a cost-effective and fast method. One can easily explore different scenarios (e.g., by means of titrations) and identify the most important parameters that determine the final speciation of a metal. In our specific case, chemical analysis was not a serious option, because of the low volumes (<1 mL) available for analysis (see Experimental Section). We have chosen to calculate the speciation in our samples in combination with bioassays. This allows quantification of toxic levels of the relevant species (i.e., the free-metal ion) at low cost and in little time.

Whereas a body of evidence exists to support the FIM for divalent metals (discussed in ref 5), information on trivalent metals is limited. Best studied are iron (13) and aluminum (14), but application of the FIM has never been described for elements of the lanthanide series. The lanthanides, ranging from lanthanum (La, $Z = 57$) to lutetium (Lu, $Z = 71$), comprise 15 homologous trivalent metals, of which 14 commonly occur in nature (15). Industrial uses and applications of lanthanides are still increasing (16) and so are the accompanying emissions to the environment. The larger part of the emissions ends up in aquatic environments (17), but little is known about the effects of lanthanides on aquatic

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biota. Therefore, lanthanides have received more attention in the past few years. Some aquatic toxicity studies were performed, but because of the low solubility products of lanthanides with phosphate and carbonate, which are present in most artificial media, substantial precipitation occurred in these experiments (e.g., refs 18 and 19). This made it difficult to relate toxic effects to total dissolved concentrations, let alone to free-ion concentrations. Another problem is that not many speciation programs have lanthanide stability constants in their database. For vertebrates it was suggested that toxicity of gadolinium (Gd, $Z = 64$) is related to the free-ion concentration (20), but Jackson et al. (21) found no correlation between Gd toxicity and free-ion concentrations. Stanley and Byrne (22) found a clear relation between concentrations of free Gd^{3+} and Gd uptake by marine algae in carbonate-manipulated media but could not completely explain their observations based on Gd^{3+} alone.

This work aims at testing the FIM for Lu, the heaviest lanthanide, in a bioassay with the luminescent bacterium *Vibrio fischeri* Beijerinck 1889 (formerly known as *Photobacterium phosphoreum*). *V. fischeri* is widely used in a non-substance-specific test system known as the Microtox system (Azur Environmental, Carlsbad, CA). Briefly, during metabolism of its substrate the bacterium is not utilizing all available ATP energy and converts a fixed portion of this energy into light (luminescence). Conversely, the extent of luminescence is proportional to the overall metabolism, whereby at lower luminescence there is lower metabolism. Hence at increasing exposure to a toxic substance, the decrease in luminescence is an indicator of toxic stress. *V. fischeri* was selected because it is possible to expose it in a simple nitrate medium, whose metal-complexing characteristics are easy to manipulate. Moreover, complexation experiments with Cd, Cu, Ni, Pb, and Zn demonstrated that toxic effects on *V. fischeri* correlate best with free-metal ions (23–26). In addition, an extensive literature search was performed to collect reliable equilibrium constants for Lu with several organic and inorganic ligands.

Experimental Section

Experiments. *Vibrio fischeri* is a marine bacterium and therefore is usually studied in NaCl solutions. However, to mimic speciation in freshwater (27), it can also be exposed in a 0.355 M (or 3.02%) $NaNO_3$ solution. While the 0.355 M ionic strength is much larger than that of freshwater, high ionic strength and presence of Na^+ are necessary for undisturbed performance of the bacteria (28). This $NaNO_3$ solution was chosen because nitrate has a weak complexing affinity for lanthanides (29) and thus has a limited influence on free-ion concentrations.

$NaNO_3$ (99.8%) was obtained from J. T. Baker. The source for Lu was an ICP stock solution (10 000 ppm of 99.999% Lu_2O_3 in 4% HNO_3 , CPI International, Santa Rosa, CA). The amount of NO_3^- that was introduced through the use of this stock was accounted for in preparing the final NO_3^- concentrations. The pH of the medium was adjusted to 5.50 ± 0.10 with HNO_3 (Merck, Suprapur) and NaOH (J. T. Baker, 98.6%), by use of a Metrohm 744 pH meter, equipped with a Solitrode – combined LL pH electrode with a Pt 1000 temperature sensor. Before each series of measurements, the pH meter was calibrated with standard pH-buffered solutions of pH 4.00 and 7.00, which cover the range of pH values encountered in this work. Amounts of added acid and base were noted to make volumetric corrections and also to account for added Na^+ and NO_3^- in the speciation calculations. All solutions were prepared in Milli-Q water (Millipore Waters, Milford, MA).

Using the above medium, we prepared two Lu series: (1) a logarithmically ordered range of Lu concentrations with no added ligands (10 concentrations between 0.15 μM and

2.56 mM) and (2) two Lu concentrations (5 and 50 μM) each with six concentrations of the following organic ligands: ethylenediaminetetraacetate (EDTA, Sigma Chemical Co., ca. 99%), nitrilotriacetate (NTA, Sigma Chemical Co., ca. 99.5%), citrate (J. T. Baker, 99.8%), malate (Acros, 99%), oxalate (Merck, 99.5%), and acetate (J. T. Baker, 99.5%). All six ligands were also tested as pure substances (i.e., with no added Lu) to ensure that ligands were not toxic over the range of concentrations utilized in the experiments. Ligands were chosen on the basis of their differences in complexing strength for Lu and their different charges in fully dissociated state: 4– for EDTA, 3– for NTA and citrate, 2– for malate and oxalate, and 1– for acetate. The speciation of each ligand and its effective charge will, of course, be determined by the degree of protonation at pH 5.50 and is accounted for in all calculations. In addition, it should be noted that citrate, malate, oxalate, and acetate are naturally occurring ligands, while EDTA and NTA in the environment are of anthropogenic origin.

A volume of 150 μL of the prepared test concentration was pipetted into white 96-well plates (Luminoscan LB 96P WMP 25). All tests were performed in triplicate. Each series had its own control, consisting of a 0.355 M $NaNO_3$ solution at pH 5.50. In each plate, a series of Cu solutions (Johnson Matthey, AAS standard solution, Specpure, 1000 ppm in 5% HNO_3) was used as positive control. The plate, filled with test solutions, was put into the luminometer, which was equipped with an automatic injector (Labsystems Luminoscan RS).

Lyophilized bacteria (*V. fischeri*, NRRL B-11177, Microtox Acute Reagent, Azur Environmental, Carlsbad, CA) were prepared for the experiments as described by Hamers et al. (30). First, the condition of the bacteria was tested by observing luminescence in the control over a period of 5 min. If the bacterial condition was satisfactory (a small increase of light emission followed by a slow decrease), 75 μL of the bacterial suspension (in 0.355 M $NaNO_3$, pH 5.50) was injected into each well of the plate. Exactly 7.5, 15, 22.5, and 30 min after injection, the luminescence of each well was measured and expressed in relative light units (RLU). This bioluminescence response provides an indirect assessment of the metabolic rate of the bacteria, and as such is a valuable endpoint (31). The chemical concentrations used in all calculations were the final concentrations in the wells after addition of the bacterial suspension. The experiments were conducted at a temperature of $20 \pm 3^\circ C$.

Because of reported problems with pH control in this biotest system (25, 32), we checked the pH at the end of the experiments by collecting the contents of three replicate wells (ca. 650 μL) in an Eppendorf vial with a pipet. The pH of the composite solution was measured with a micro pH meter (Knick 646, equipped with a glass electrode), and the pH of the bacterial suspension was also measured.

Chemical Equilibrium Program CHEAQS. For the speciation calculations we chose the program CHEAQS (33), because it has an extensive and well-documented database (including Lu, Cu, acetate, EDTA, and NTA). A background document is available containing all equilibrium constants, literature references, and the required conversions. The database is easily changed or extended with user-friendly routines. This free program and the background document can be downloaded from the Internet (<http://home.tiscali.nl/cheaqs/>).

The program settings for the equilibrium calculations were as follows: solids were allowed to precipitate in case of oversaturation (to assess saturation states); CO_2 saturation of the solution was assumed (by use of a pCO_2 of 3.5×10^{-4} atm, corresponding to the ambient concentration of this gas), which in turn, with pH, determined the concentration of free CO_3^{2-} in solution (in a laboratory, the pCO_2 may be

TABLE 1. Cumulative Stability Constants ($\log_1 \beta_n$) and Solubility Products ($-\log K_s$) for Lu, Appropriate at 25 °C^a

complex	$\log_1 \beta_n$ $I = 0.0 \text{ M}$	$\log_1 \beta_n$ $I = 0.34 \text{ M}$	ref
Lu(OH) ²⁺	6.727 ^b	5.915	40
Lu(OH) ₂ ⁺	12.324 ^b	10.971	36
Lu(OH) ₃	18.141 ^b	16.518	36
Lu(OH) ₄ [−]	24.05	22.43	37
Lu ₂ (OH) ₂ ⁴⁺	14.60	14.06	estimated value ^c
Lu(CO ₃) ⁺	7.75	6.13	38
LuH(CO ₃) ₂ ⁺	12.819 ^d	11.466	38
Lu(CO ₃) ₂ [−]	13.37	11.21	38
Lu(NO ₃) ₂ ⁺	−0.076	−0.888	29
Lu(Ac) ₂ ⁺	2.491 ^e	1.679	10
Lu(Ac) ₂ ⁺	4.228 ^e	2.875	10
Lu(Ac) ₃	5.301 ^e	3.678	10
Lu(NTA)	14.242 ^e	11.807	10
Lu(NTA) ₂ ^{3−}	23.572 ^e	21.137	10
Lu(NTA)(OH) [−]	20.542 ^{e,f}	18.107	10
Lu(EDTA) [−]	22.303 ^e	19.056	10
Lu(Cit)	9.670	7.235	10
Lu(Cit) ₂ ^{3−}	16.22 ^e	13.79	35
Lu(Mal) ⁺	6.361 ^e	4.738	10
Lu(Mal) ₂ [−]	10.379 ^e	8.215	10
Lu(Ox) ⁺	6.96	5.34	41
LuH(Ox) ₂ ⁺	7.052 ^g	5.699	41
Lu(Ox) ₂ [−]	11.77	9.61	41
Lu(Ox) ₃ ^{3−}	13.959 ^h	12.336	10
Lu(Ox) ₄ ^{5−}	14.680 ^h	14.680	10

solid	$-\log K_s$ $I = 0.0 \text{ M}$	$-\log K_s$ $I = 0.34 \text{ M}$	ref
Lu(OH) ₃	25.00	23.38	39
Lu ₂ (CO ₃) ₃	30.80	26.74	estimated value ^c

^a Constants are defined as $\beta_n = [\text{Lu}_a\text{H}_b\text{L}_n]/([\text{Lu}]^a[\text{H}]^b[\text{L}]^n)$ for the reaction $a\text{Lu} + b\text{H} + n\text{L} \leftrightarrow \text{Lu}_a\text{H}_b\text{L}_n$ where $a = 1$ or 2 , $b = 0$ or 1 , and $n = 1, 2, 3$, or 4 . Ac = acetate, Cit = citrate, Mal = malate, Ox = oxalate, Ln = lanthanide. ^b Calculated from hydrolysis constants by use of a $\log K_w$ of 13.997 at $I = 0.0 \text{ M}$ (10). ^c By use of Ln data ($n = 7$) from ref 10. ^d Converted to cumulative stability constants by use of a $\log \beta$ of 10.329 for HCO_3^- at $I = 0.0 \text{ M}$ (10). ^e Converted from $I = 0.1 \text{ M}$ with the Davies equation. ^f Constant defined as $\beta = [\text{Lu(NTA)(OH)}^-]/([\text{Lu}^{3+}][\text{NTA}^{3-}][\text{OH}^-])$. ^g Converted from $I = 0.05 \text{ M}$ with the Davies equation after conversion to cumulative stability constants, by use of a $\log \beta$ of 3.92 for H(Ox)^- at $I = 0.05 \text{ M}$ (41). ^h Converted from $I = 1.0 \text{ M}$ with the Davies equation, determined at 20 °C.

higher due to the presence of experimenters, but since our experiment was performed within a closed luminometer, this effect was deemed negligible); ionic strength was calculated rather than fixed; redox equilibria were not included (because they were not relevant under the prevailing conditions); pH was entered (measured for each test concentration); for all other components, the added total concentration (M) was entered; the convergence criterion was set to 1×10^{-6} (0.0001%).

Equilibrium Constants. Most of the equilibrium constants in the CHEAQS database are taken from Martell and Smith (10) and some from Turner et al. (34). However, where we felt certain that recent literature of high quality was not yet included in the database, we added or replaced equilibria (29, 35–41). When necessary, $\log K$ values were converted from stepwise to cumulative stability constants (i.e., $\log \beta$ values) and corrected to infinite dilution ($I = 0.0 \text{ M}$) by use of the Davies equation in CHEAQS. Two complexation constants [$\text{Lu}_2(\text{OH})_2^{4+}$ and $\text{LuH}(\text{EDTA})$] and the solubility product for $\text{Lu}_2(\text{CO}_3)_3$ were not found in the above-mentioned sources. Of these, the complex $\text{LuH}(\text{EDTA})$ is negligible at pH 5.5. Values for the other two equilibria were estimated from available data on other lanthanides (taken from ref 10) by extrapolating a smooth curve. Table 1 lists all the Lu complexation constants and solubility products we used, as

both intrinsic ($I = 0.0 \text{ M}$) and conditional ($I = 0.34 \text{ M}$) equilibrium constants, and includes notes on the conversions made. In collecting the stability constants, we tried to be as complete as possible, although some of the Lu complexes are likely to turn out negligible. This, however, is difficult to predict beforehand. A value for the solubility product of $\text{Lu}_2(\text{Ox})_3$ could neither be found in the literature nor estimated from available data ($n = 4$). However, precipitation of $\text{Lu}_2(\text{Ox})_3$ was not expected in our experiments, since the product $[\text{Lu}]^2[\text{Ox}]^3$ was always below the values for other lanthanide–oxalate solubility products (9). For citrate, oxalate, and malate, which are not present in the standard database of CHEAQS, we also added stability constants (taken from ref 9) for the association of H^+ and Na^+ with these ligands.

Data Handling and Statistics. For each exposure period the response data, that is, relative light units (RLU), were converted to percentages of the control response, of which the average ($n = 3$) was set to 100%. Concentration–response relations were described with the Weibull model (42, 43), given by

$$R_C = \frac{R_0}{2^{(C/\text{EC}_{50})^b}} \quad (1)$$

wherein R_C is the performance as a function of the concentration (in percent of control performance), R_0 is the control performance (a constant, set to 100%), C is the final Lu concentration in the well (μM), EC_{50} is the Lu concentration for which the response is 50% (μM), and b is the dimensionless shape (slope) parameter of the concentration–response curve. To be able to compare our results with those from other toxicity studies, EC_{50} calculations are primarily based on free Lu^{3+} concentrations instead of activities. However, any conclusion based on concentrations is equally valid for activities, because the ionic strength of our solutions was constant ($I = 0.34 \text{ M}$). Free Lu^{3+} concentrations may be converted to activities by multiplying concentrations by the activity coefficient $\gamma_{\text{Lu}^{3+}} = 0.0607$ (as calculated with the Davies equation in CHEAQS).

By use of the EC_{50} values of the four exposure times (7.5, 15, 22.5, and 30 min), the EC_{50} at infinite exposure time and the elimination rate constant k were calculated (44, 45):

$$\text{EC}_{50,t} = \frac{\text{EC}_{50,\infty}}{1 - e^{-kt}} \quad (2)$$

wherein $\text{EC}_{50,t}$ is the EC_{50} as a function of exposure time (μM), $\text{EC}_{50,\infty}$ is the EC_{50} at infinite exposure (μM), k is the elimination rate constant (min^{-1}), and t is exposure time (min).

All linear and nonlinear least-squares regressions were performed with the software package Prism, release 2.01 (GraphPad Software, San Diego, CA).

Results and Discussion

Toxicity of Lu in the Absence of Organic Ligands. The toxicity of Lu from the experiments in the absence of organic ligands was expressed in terms of both calculated free Lu^{3+} and total dissolved Lu concentrations. The difference between these two measures is attributable to the complexation of Lu by nitrate, hydroxide, and carbonate (about 3.7%, 0.34%, and 0.02%, respectively). Consequently, the concentration–response curves and calculated $\text{EC}_{50,t}$ values for total dissolved Lu and free Lu^{3+} at each of the four exposure times are very close (Table 2). Table 2 also shows that the (relative) standard error of the $\text{EC}_{50,t}$ decreases with increasing exposure times. This is probably related to Lu accumulation not having reached equilibrium in all bacteria when the first measure-

TABLE 2. Values for $EC_{50,t}$ and Elimination Rate $k \pm$ Standard Error for Free Lu^{3+} Concentrations and Total Dissolved Lu Concentrations in the Presence and Absence of Organic Ligands^a

experiment	$EC_{50,t}$ (μM)					k (min^{-1})
	7.5	15	22.5	30	∞	
Lu^{3+} no ligand	2.16 ± 0.10	1.57 ± 0.05	1.44 ± 0.04	1.37 ± 0.04	1.35 ± 0.01	0.131 ± 0.003
Lu^{3+} EDTA	0.86 ± 0.03	0.80 ± 0.03	0.80 ± 0.04	0.80 ± 0.05	0.799 ± 0.001	0.353 ± 0.006
Lu^{3+} NTA	1.77 ± 0.09	1.34 ± 0.13	1.00 ± 0.16	0.74 ± 0.17	0.77 ± 0.18	0.073 ± 0.027
Lu^{3+} citrate	$2.29 \pm nc$	$2.23 \pm nc$	$2.19 \pm nc$	1.95 ± 0.20	2.11 ± 0.09	0.33 ± 0.12
Lu^{3+} malate	5.02 ± 0.71	3.02 ± 0.44	1.95 ± 0.25	1.28 ± 0.13	0.55 ± 0.50	0.015 ± 0.015
Lu^{3+} oxalate	5.73 ± 0.36	2.58 ± 0.03	2.31 ± 0.02	2.15 ± 0.04	1.11 ± 0.65	0.030 ± 0.021
total Lu no ligand	2.27 ± 0.11	1.64 ± 0.05	1.51 ± 0.04	1.43 ± 0.04	1.41 ± 0.01	0.131 ± 0.003
total Lu EDTA	2.36 ± 2.47	1.16 ± 1.40	1.02 ± 1.26	0.96 ± 1.19	0.62 ± 0.20	0.042 ± 0.017
total Lu NTA	11.4 ± 5.5	6.85 ± 3.87	5.44 ± 3.04	4.54 ± 2.40	3.66 ± 0.15	0.051 ± 0.003
total Lu citrate	40.5 ± 21.7	37.5 ± 32.9	40.4 ± 48.9	43.2 ± 62.2	40.4 ± 1.6	0.79 ± 3.9
total Lu malate	42.9 ± 25.9	32.5 ± 23.0	26.9 ± 23.9	19.5 ± 19.3	21.4 ± 3.8	0.088 ± 0.028
total Lu oxalate	36.2 ± 10.7	32.3 ± 14.5	31.5 ± 19.9	31.8 ± 26.8	31.7 ± 0.1	0.277 ± 0.007

^a Calculated with eqs 1 and 2; nc, not calculable.

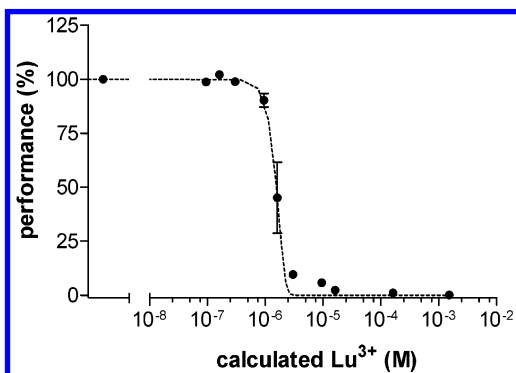


FIGURE 1. Luminescence in percentage of control vs calculated free Lu^{3+} concentrations, after 15 min of exposure. Symbols are averages ($n = 3$) with standard error bars. If error bars are not visible, they fall within the symbols. Dotted line is a fit ($r^2 = 0.97$) of eq 1. The control treatment without Lu is plotted on the left segment of the x-axis.

ments were made, causing variation in responses. This coincides with the increase in the value of b , the slope of the concentration–response curve (data not shown; for a discussion on slope values see ref 46). Figure 1 gives the concentration–response curve for free Lu^{3+} after 15 min of exposure. It shows that the slope of the curve is very steep, indicating some sort of threshold at approximately $1 \mu M$, above which the performance is strongly inhibited. Comparison with other metals, studied under the exact same conditions by McCloskey et al. (27), showed that free Lu^{3+} is more toxic than, for instance, free Cd^{2+} and Zn^{2+} but less toxic than free Pb^{2+} ($EC_{50,15}$ values \pm standard errors are 19 ± 4 , 28 ± 6 , and $0.46 \pm 0.05 \mu M$, respectively). In the same study, an $EC_{50,15} \pm$ standard error for free La^{3+} of $322 \pm 31 \mu M$ was reported, which implies that free Lu^{3+} is approximately 200 times more toxic than the first member of the lanthanide series.

Figure 2 shows the $EC_{50,t}$ for free Lu^{3+} , from which the $EC_{50,\infty}$ and the elimination rate constant k were calculated with eq 2. Values for these parameters are given in Table 2. Again, the $EC_{50,\infty}$ values for free Lu^{3+} and total dissolved Lu are very similar, and the value for the elimination rate constant k was exactly the same: $0.131 \pm 0.003 min^{-1}$. It is noted that the models from eqs 1 and 2 agreed very well with the data.

Toxicity of Ligands and of Cu. Experiments with the pure ligands showed no toxicity in the concentration ranges tested, except for acetate. In Figure 3, the data for acetate and EDTA are given, the latter as an example of the five ligands used

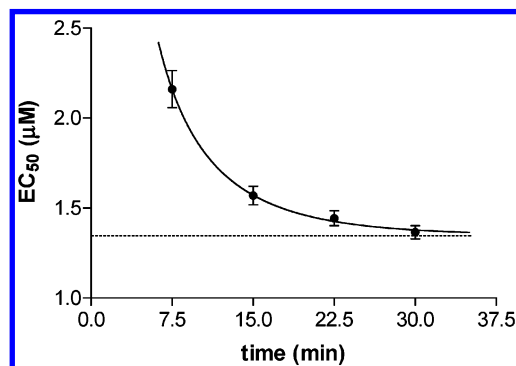


FIGURE 2. EC_{50} for calculated free Lu^{3+} concentrations vs exposure time. Symbols are $EC_{50,t}$ values with standard error bars, calculated with eq 1. The solid line is a fit ($r^2 = 0.999$) of eq 2 and the dotted line is the calculated $EC_{50,\infty}$ (μM), given in Table 2.

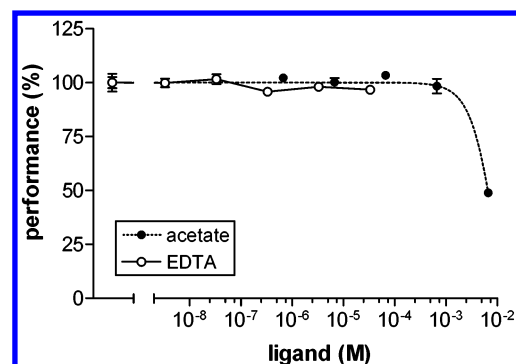


FIGURE 3. Luminescence in percentage of control vs EDTA (○) and acetate (●) concentrations after 15 min of exposure. Symbols are averages ($n = 3$) with standard error bars. If error bars are not visible, they fall within the symbols. The dotted line through the acetate observations is a fit ($r^2 = 0.96$) of eq 1. The control treatment without ligand is plotted on the left segment of the x-axis.

in the complexation experiments. At concentrations above 1 mM, acetate has toxic effects on *V. fischeri* ($EC_{50,15} \pm$ standard error is $6.54 \pm 0.26 mM$). However, these high acetate concentrations would be needed to complex Lu and hence be able to test the FIM; otherwise there is hardly any difference between total dissolved Lu and free Lu^{3+} concentrations. Consequently, acetate was excluded from the Lu ligand experiments, because its complexing intensity (see Table 1) is too low at nontoxic concentrations. The FIM could not be studied with this ligand.

The $EC_{50,15} \pm$ standard error for free Cu^{2+} , which is the positive control in our experiments, was $1.04 \pm 0.17 \mu M$ (r^2

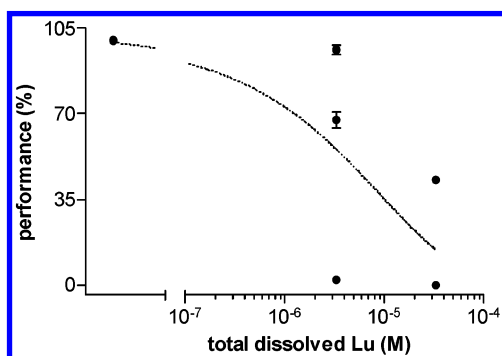


FIGURE 4. Luminescence in percentage of control vs total dissolved Lu concentrations after 30 min of exposure in the presence of NTA. Symbols are averages ($n = 3$) with standard error bars. If error bars are not visible, they fall within the symbols. The dotted line is a fit ($r^2 = 0.51$) of eq 1. The control treatment without Lu is plotted on the left segment of the x -axis.

$= 0.96$). This value is in good agreement with the value of $1.23 \pm 0.10 \mu\text{M}$, found by McCloskey et al. (27) under nearly the same conditions, namely, in a 0.355 M NaNO_3 medium of pH 5.35 after 15 min of exposure. The outcome of this positive control experiment implies a good physiological quality of the bacteria and, more importantly, attests to the reproducibility of this biotest.

Toxicity of Lu in the Presence of Organic Ligands.

Concentration–response curves (eq 1) were fit to the data from the Lu–organic complexation experiments: first, on the basis of total dissolved Lu concentrations, and second, on the basis of calculated free Lu^{3+} concentrations. In case Lu toxicity follows the FIM, then concentration–response curves, based on free Lu^{3+} concentrations, will better fit to the data than curves based on total dissolved Lu. Furthermore, compliance with the FIM will yield comparable EC_{50} values when results are expressed in free Lu^{3+} concentrations.

As expected, for total dissolved Lu concentrations the fit to the data was poor (r^2 ranged from 0.11 to 0.51). Figure 4 shows the best fit of this series ($r^2 = 0.51$), which occurred for Lu in the presence of NTA after 30 min of exposure. At total Lu concentrations of 3.3 and $33 \mu\text{M}$ in the presence of organic ligands, the full range of responses between 0 and 100% is possible (see Figure 4), suggesting no relation between total dissolved Lu concentrations and performance. In Table 2, the $\text{EC}_{50,t}$, $\text{EC}_{50,\infty}$, and k values, based on total dissolved Lu, are presented.

The performance observations shift toward a much more apparent relation with concentration, when the latter is expressed as free Lu^{3+} , and the fits improve significantly (r^2 ranges from 0.87 to 0.996). In Figure 5, data are presented for each of the five tested ligands, after 15 min of exposure. Table 2 gives the values for $\text{EC}_{50,t}$ for all four exposure times, for calculated free Lu^{3+} concentrations. Also, the values for $\text{EC}_{50,\infty}$ and k are given. Figure 6 shows the EC_{50} values for free Lu^{3+} against time and the fits of eq 2 to these data.

The $\text{EC}_{50,t}$ values based on total dissolved Lu have large standard errors, while those based on free Lu^{3+} were estimated with much better precision (except for the EC_{50} values at $t = 7.5, 15$, and 22.5 min in the citrate experiment). For citrate it should be noted that there is no consensus yet on the nature of the lanthanide complexes that this ligand forms (47). Therefore, the high variation in the Lu–citrate data may partly be due to uncertainties in the speciation calculations. The $\text{EC}_{50,t}$ values in Table 2 range from 0.55 to $5.73 \mu\text{M}$ for free Lu^{3+} and from 0.62 to $43.2 \mu\text{M}$ for total dissolved Lu. The ranges of these values already hint at the importance of the free Lu^{3+} ion. For a better comparison of the different experiments, the $\text{EC}_{50,t}$ values for $t = 30$ min are used, since at this time the EC_{50} will have reached a stable

value. For free Lu^{3+} , the values range from 0.74 to $2.15 \mu\text{M}$, and for total dissolved Lu, from 0.96 to $43.2 \mu\text{M}$. Thus, the maximum difference is a factor of 2.9 and 45, respectively, which further supports the role of the free ion in determining toxicity.

The EC_{50} values, obtained in the Lu–organic complexation experiments, show the same trend as for Lu in the absence of organic ligands: in time they decrease. The rate at which the EC_{50} decreases with time is determined by the elimination rate constant k . This parameter may be regarded as a desorption rate constant, describing the release of Lu from the bacterial cell. Thus, when k is high, the bacteria rapidly achieve equilibrium with Lu in solution and EC_{50} values will hardly change over time (see, for instance, EDTA in Figure 6). For the two weakest natural organic ligands, malate and oxalate, a distinct decrease of the EC_{50} with time is noted (Figure 6), caused by the small elimination rate constants in these experiments (Table 2). Hence, in the presence of malate or oxalate, an equilibrium between free Lu^{3+} in solution and the bacteria is reached more slowly than for EDTA, NTA, and citrate or in the absence of organic ligands. This is likely related to complexation (kinetics) differences of the used media, but how exactly is as yet unknown. These observations further underline the importance of comparing toxicity data only for a distinct point-in-time, preferably when some sort of equilibrium is achieved (in our case after about 30 min). Otherwise, one might conclude that at $t = 7.5$ min there is no compliance with the FIM, whereas at $t = 30$ min the opposite is the case.

EC_{50} values based on free Lu^{3+} converge to a value of around $1.5 \mu\text{M}$ (Figure 6), while the $\text{EC}_{50,\infty}$ for Lu^{3+} in the absence of organic ligands is $1.35 \mu\text{M}$ (Table 2). On basis of these observations and the aspects discussed above, we conclude that for Lu (and most likely also for other members of the lanthanide series) the FIM is valid. While this conclusion is based on free Lu^{3+} concentrations, it is also valid for Lu^{3+} activities, because all solutions were of the same and constant ionic strength ($I = 0.34 \text{ M}$), in which case the Lu^{3+} activity is directly proportional to the Lu^{3+} concentration. The observed (small) deviations of the EC_{50} values from the expected value, derived from the Lu experiment in absence of organic ligands, may be caused by violations of the assumptions that underlie the FIM. These include (1) contributions to the observed toxicity from metal species other than the free ion; (2) shortcomings in the speciation calculations, for example, inaccurate $\log \beta$ values, missing constants, imprecise concentrations; and (3) significant nonequilibrium effects, such as bacterial growth.

Also, it must be noted that because of the equilibrium $\text{OH}\beta_1 = [\text{Lu}(\text{OH})^{2+}][\text{Lu}^{3+}]^{-1}[\text{OH}^{-}]^{-1}$ and the constancy of pH, thus constant OH^{-} concentration, the ratio of free Lu^{3+} and $\text{Lu}(\text{OH})^{2+}$ concentrations is fixed. This means that the concentration–effect curve based on $\text{Lu}(\text{OH})^{2+}$ has the same shape and statistically an equally good fit as the one based on free Lu^{3+} (shown in Figure 5). The same holds for other OH^{-} (i.e., pH-) dependent Lu species and in fact also for Lu–nitrate and Lu–carbonate species, because the NO_3^{-} concentration and CO_2 partial pressure are constants as well. Consequently, our experiments cannot discriminate between effects of free Lu^{3+} and other inorganic Lu species that are present in constant proportion to free Lu^{3+} . This statement holds for many experiments that were originally designed to test the FIM. Also, the dissociation rate of inorganic metal complexes is generally considered to be much higher than for organic complexes (6), and the former therefore appear to be available for uptake. For this reason, in Fe^{3+} studies, the concept of Fe' is used, comprising all dissolved inorganic $\text{Fe}(\text{III})$ species considered available for uptake by algae (6, 13, 48). For Lu we may conclude that species responsible for the effect are at least proportional to the free Lu^{3+} concen-

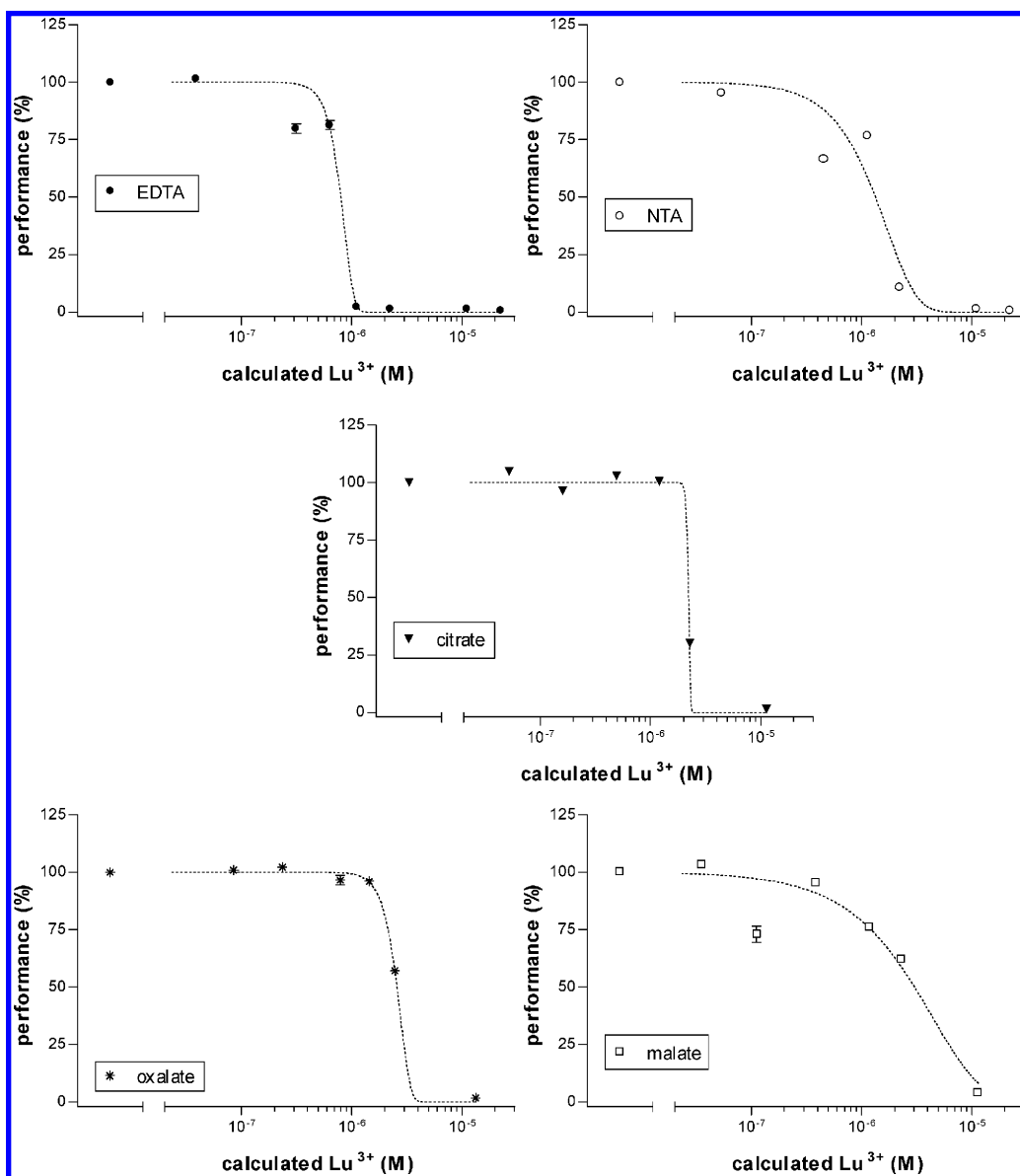


FIGURE 5. Luminescence in percentage of control vs calculated free Lu^{3+} concentrations in the presence of each of the five tested ligands after 15 min of exposure. Symbols are averages ($n = 3$) with standard error bars. If error bars are not visible, they fall within the symbols. The dotted lines are fits of eq 1. The control treatment without Lu is plotted on the left segment of the x-axes.

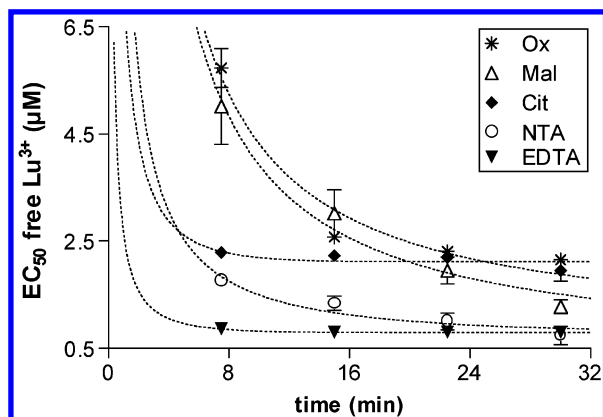


FIGURE 6. EC_{50} for calculated free Lu^{3+} concentrations vs exposure time. Symbols are $\text{EC}_{50,t}$ values with standard error bars, calculated with eq 1. The dotted lines are fits of eq 2.

tration. Furthermore, Figure 7 shows that the concentrations of other inorganic species around pH 5.5 are low compared

to that of free Lu^{3+} , suggesting a dominant role for the latter in establishing the observed effects. Still, a concept such as Lu' , to denote free Lu^{3+} plus all potentially available inorganic Lu species present at fixed ratios to Lu^{3+} , could prove very useful in lanthanide studies.

Influence of pH. Measurements of pH revealed that it shifted upward during the experiments from the initial 5.50 ± 0.10 to an average value of 6.30 ± 0.39 . This is apparently due to the activity of the bacteria, because the bacterial suspension, which was also prepared with a NaNO_3 solution of pH 5.5 (see Experimental Section), had an average pH of 6.95 ± 0.12 at the end of the experiment. Because pH is one of the major factors affecting metal speciation, we studied how this pH shift would alter the speciation of Lu in our system.

Figure 7 presents the speciation of $3.33 \mu\text{M}$ Lu in a 0.355 M NaNO_3 medium as a function of pH (titration calculated with CHEAQS). The figure shows that in the pH range 4.50–6.50 there is virtually no change, and free Lu^{3+} is the dominant species. Speciation calculations were also made for media containing organic ligands ($10 \mu\text{M}$ for EDTA, NTA, and citrate and $100 \mu\text{M}$ for oxalate and malate) and $3.33 \mu\text{M}$ Lu, in the

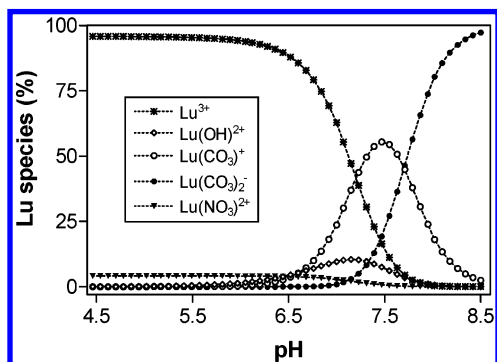


FIGURE 7. Lu (3.33 μ M) speciation in 0.355 M NaNO₃ medium as a function of pH, at $p\text{CO}_2 = 3.5 \times 10^{-4}$ atm, calculated with CHEAQS.

pH range 5.5–6.3. The calculations showed that for malate and oxalate there was no significant change in free Lu³⁺ concentrations, but for EDTA, NTA, and to a lesser extent for citrate, the free Lu³⁺ concentration decreased with increasing pH. However, the outcome of these calculations strongly depends on the ratio between the Lu concentration and that of the ligand. Anticipating a gradual increase of pH in the course of the experiments, we consider the results based on the observations at 7.5 and 15 min robust. Data obtained at 22.5 and 30 min may be less reliable for they approach pH values at which Lu complexation starts to shift and other species, that is, Lu(CO₃)⁺, start to form.

Minimization of pH shifts requires the addition of a pH buffer. However, this means the introduction of a new component in the NaNO₃ medium, which must be included in the speciation calculations. Since most pH buffers have some affinity for metals as well, they will complex a certain part of the free-metal ions in solution. To estimate how much, stability constants of metals with buffers are needed, but these are seldom available or reliable (and for Lu these values are nonexistent). In addition, a pH buffer will also influence speciation by changing the ionic strength of the solution. With respect to the complexing capacity and the pH chosen, the best candidate for our system would have been the Good buffer 2-morpholinoethanesulfonic acid (MES) (49). Considering the aforementioned factors, and also taking the limited pH increase in our experiments into account, which did not strongly influence Lu speciation, we feel that not introducing a buffer is justified. However, for other metals, medium compositions and/or different pH values the application of a buffer might be necessary. This should be judged in terms of speciation uncertainties caused by use of poorly characterized buffers, on one hand, and pH variations on the other.

Additionally, pH changes will influence the surface charge of the bacteria. In our case the pH increased, which results in a net deprotonation of ligands at the cell surface and may enhance the binding of Lu³⁺. However, with an increase in solution pH, the concentration of free Lu³⁺ will decrease (see Figure 7), so that these forces will counteract. Taking into account the limited increase in pH in the present study, we consider these effects insignificant.

In short, the data described in this paper support the FIM for Lu effects on the luminescence response of *V. fischeri*. The toxicity of Lu³⁺ is higher than those of Cd²⁺ and Zn²⁺ but comparable to that of Cu²⁺. Adverse effects of Lu occurred at concentrations that exceed natural surface water levels by 2–3 orders of magnitude (15). Compared to the EC₅₀ of La, the lightest lanthanide, Lu is 200 times more toxic. The exact cause for this difference, which is probably related to the much larger stability constants of Lu relative to La, is still unknown and will be the focus of future research.

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